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(54) Title: SKIN LIGHTENING AGENTS

(57) Abstract: The invention relates to the use of vinylguaiacol and/or ethylguaiacol as a skin lightening agent in combination with an agent which decreases the cytotoxicity of vinylguaiacol and/or ethylguaiacol on skin cells. Thus, the invention provides a skin lightening composition with decreased cytotoxic effects against skin cells and an agent.

SKIN LIGHTENING AGENTS

The present invention relates to the use of vinylguaiacol and/or ethylguaiacol as a skin lightening agent in combination with an agent which decreases the cytotoxicity of vinylguaiacol and/or ethylguaiacol on skin cells.

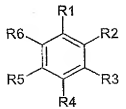
There are three main uses for skin lighteners; to lighten age spots (liver spots or senile lentigo), to reduce the brown-black colour of non-Caucasian skin and to prevent the darkening of Caucasian and Japanese skins. The main mechanism of action is believed to involve inhibition of the enzyme tyrosinase that is involved in the metabolism of tyrosine into melanin in the melanocytes present in the skin. Therefore, to be effective, it is desirable that a skin lightener should inhibit mammalian tyrosinase effectively, should be permeable through both the cell and melanocyte membranes and should be safe and non-toxic to skin cells at the concentration it is to be used at.

Commonly used skin lighteners are hydroquinone, arbutin and kojic acid. Hydroquinone is the most effective but has significant adverse effects so that it is no longer registered for use in the EC. Many other materials have also been used or documented as skin lighteners, including lactic acid, ferulic acid and nicotinamide as well as plant extracts such as Bearberry extract. Other skin lighteners include glycosides and esters of hydroxysalicylic acid, ascorbyl methylsilanol and liquorice extracts. The use of caffeic acid or an ester or amide thereof to depigment the skin is described in US 5164185. Depigmenting compositions containing di- or tri- caffeoylquinic acid are disclosed in US 5445816. Vinylphenol or vinylsyringol (analogues of vinylguaiacol) do not show the effects of the present invention.

The naturally occurring compound ferulic acid is known to have skin lightening activity. Ferulic acid is a phenylpropenyl molecule.

Known skin lighteners, such as vinylguaiacol or ethylguaiacol may exhibit some cytotoxicity against skin cells. Whilst such cytotoxicity can be tolerated, it would be advantageous to ameliorate it to some extent. The present invention addresses this issue.

According to the first aspect of the invention, there is provided a composition comprising a compound of formula I and an agent, wherein the agent decreases the cytotoxicity of the compound of formula I on skin cells:



I

wherein R^1 is $CH=CH_2$, R^3 is $-OCH_3$; R^4 is OH; and R^2, $R^5$and R^6 are all hydrogen (vinylguaiacol) or R^1 is CH_2-CH_3; R^3 is $-OCH_3$; R^4 is OH; and R^2, $R^5$and R^6 are all hydrogen (ethylguaiacol). The composition is preferably for use as a skin or hair lightener. The compound of formula I can be provided as a salt, for example as a sodium or potassium salt.

The compound of formula I acts as a skin lightening agent. For the purposes of this invention, the term "skin lightener" or "skin lightening agent" includes an agent which inhibits melanogenesis, especially the enzymes (such as tyrosinase) which are associated with the formation of melanin by mammalian cells, such as melanocytes in the skin.

The compound of formula I for the purposes of this invention, is provided for a number of different cosmetic and/or therapeutic uses. Examples of such uses are to maintain a light or fair complexion, to reduce generalised over pigmentation, to lighten age spots, to lighten scars and to lighten hair.

In a preferred feature of this invention, the composition of the first aspect of the invention is applied topically to the skin or hair.

The compound of formula I is provided at levels which will inhibit melanogenesis, more specifically which cause the inhibition of enzymes associated with the formation of melanin such as tyrosinase. For the purposes of this invention, a level of a skin lightener, which produces 80% inhibition of melanogenesis, is defined as a therapeutic or cosmetically useful dose.

A compound of formula I can exhibit one or more useful activities selected from antioxidant, antimicrobial, antibrowning, aroma/flavour and acidulant activities. Skilled persons will appreciate that the antibrowning and skin lightening activities of a compound of formula I are linked. Without being bound in any way by scientific theory, they appear to work by inhibiting enzymes associated with coloured material formation. For example, the compound inhibits polyphenol oxidase (tyrosinase and laccase) enzymes which catalyse formation of brown-black pigments (melanoids) formed by oxidative polymerization of plant phenols such as chlorogenic acid in plant tissue. Similarly, the compound inhibits tyrosinase enzyme in the skin to prevent melanin formation from precursor materials present in skin cells, such as tyrosine.

A compound of the formula I can be used in a wide range of applications because of their versatility. A particular advantage of a compound of formula I is that it displays solubility in both oil and water for ease of formulation.

A still further advantage of a compound of formula I is that it possesses a pleasant aroma and this may avoid the need for the separate addition of a perfume in various compositions and formulations. Vinylguaiacol and ethylguaiacol have a fresh camphoraceous/herbal/medicated character bringing to mind some pharmaceutical cough preparations. Thus, they convey hygiene, cleanliness and well being. Vinylguaiacol and ethylguaiacol possess both a pleasant aroma and antimicrobial activity, making them especially suitable for use in formulations for application to the skin.

A compound of formula I is provided in combination with an agent which decreases the cytotoxicity of the compound of formula I on skin cells preferably increasing the viability of the skin and cells. A compound or composition which exhibits cytotoxicity against skin cells is one which causes damage to cells and may ultimately result in cell death. For the purposes of this invention, a composition or a compound (which may be vinylguaiacol and/or ethylguaiacol or a composition comprising vinylguaiacol and/or ethylguaiacol) which produces 50% cytotoxicity against skin cells is defined as a cytotoxic dose.

The agent can decrease the cytotoxicity of the compound of formula I on skin cells in at least two ways. A first way is by adding an agent which makes the cells less susceptible to the cytotoxic effects of the compound or which repairs any damage caused to the cells by the compound of formula I. As a consequence, an increased dose of the compound of formula I can be included to give the same cytotoxic effect as a previous lower dose without the agent.

Alternatively, an agent can decrease the cytotoxic effect of the compound of formula I on skin cells by increasing the efficacy of the compound as an inhibitor of melanogenesis. If the dose of the compound required to produce 80% inhibition of melanogenesis is reduced to a much lower concentration, this reduces the risk of cytotoxicity to the skin cells. An agent may achieve this

result by working synergistically with the compound of formula I, or by providing separate/unrelated skin lightening effects.

When vinylguaiacol is used as a skin lightener, the dose (concentration) of vinylguaiacol which produces 50% cytotoxicity against skin cells (cytotoxic dose) is approximately ten times greater than the dose (concentration) which produces 80% inhibition of melanogenesis (therapeutic dose). Thus, this invention provides a composition comprising vinylguaiacol and an agent wherein the difference between the inhibition of melanogenesis (preferably 80% inhibition of melanogenesis) and cytotoxicity (preferably 50% cytoxicity) on skin cells is more than 10 fold (or times). Preferably in this invention, the agent will increase the difference between the toxic dose and the therapeutic dose by between 20 and 200 times, preferably between 30 and 150 times more preferably more than 100 times.

The compound of formula I and the agent can be provided in the composition in varying concentrations and ratios. For example, the ratio of the compound of formula I and the agent in the composition can be from about 100:1 to about 1:100, preferably from 10:1 to 1:10. More preferably, ratio of the compound of formula I and agent is about 1:1.

Preferably, the agent which decreases the cytotoxicity of vinylguaiacol on skin cells, is an antioxidant. For the purposes of this invention, an anti-oxidant is any compound or substance, which inhibits or slows the rate of oxidative reactions. Examples of antioxidants include ascorbic acid, tocopherols (including alpha-tocopherol), carotenoids (including beta-carotene), ferulic acid, selenium, butylated hydroxytoluene, butyrated hydroxyanisole, ascorbyl esters (including ascorbyl palmitate) caffeic acid, propyl gallate, tertiarybutylhydroxyquinone (TBHQ) flavanoids, flavanols flavenoids and ubiquinone. An antioxidant for the purposes of this invention may be provided

in an isolated or purified form. Alternatively, the antioxidant may be provided by a plant extract. Examples of plant extracts providing antioxidants include but are not limited to palm oil or rosemary extract.

More preferably, the agent exhibits both antioxidant and skin lightening properties. Such an agent will decrease the cytotoxicity of the compound of formula I while contributing either independently or synergistically to the skin lightening effect. Examples of agents with both antioxidant and skin lightening properties include ferulic acid and ascorbic acid.

Most preferably, the agent which decreases the cytotoxicity of a compound of formula I on skin cells is one or more of tocopherol, ferulic acid or ascorbic acid. Preferably, the composition comprises two or more of tocopherol, ferulic acid and ascorbic acid in combination with a compound of formula I.

The tocopherol can be one or more selected from alpha-, beta-, gamma- or delta-tocopherol. In addition the tocopherol can be the D or the L isomer or a mixture of D and L isomers including a racemic mixture. Alternatively the tocopherol can be provided by a mixture of two or more of alpha-, beta-, gamma-, or delta-tocopherol where the tocopherol can be the D or L isomer or a mixture of D and L isomers (including a racemic mixture).

In accordance with the first aspect of the invention, the composition may comprise vinylguaiacol, ferulic acid and alpha-tocopherol. Each of the components can be provided in varying concentrations and ratios. The relative ratios of any two of the components may preferably vary from 100 to 1 fold, preferably 10 to 1 fold. The components may be provided in approximately a 1:1:1 ratio. Preferably no one component is present at a concentration of more than 100 times any other one of these components.

Alternatively, the composition may comprise vinylguaiacol, ferulic acid and ascorbic acid. The relative ratios of the components may vary from 100 to 1 fold, preferably 10 to 1 fold. The components may be provided in approximately a 1:1:1 ratio.

The composition may comprise ethylguaiacol, ferulic acid and alpha-tocopherol in varying concentrations and ratios. The relative ratios of the components may preferably vary from 100 to 1 fold, preferably 10 to 1 fold. The components may be provided in approximately a 1:1:1 ratio. Preferably no one component is present at a concentration of more than 100 times any other one of these components. Alternatively, the composition may comprise ethylguaiacol, ferulic acid and ascorbic acid. The relative ratios of the components may vary from 100 to 1 fold, preferably 10 to 1 fold. The components may be provided in approximately a 1:1:1 ratio. Preferably no one component is present at a concentration of more than 100 times any other one of the components

In accordance with the first aspect of the invention the composition may comprise vinylguaiacol and ethylguaiacol in combination with one or more of tocopherol, ferulic acid and ascorbic acid.

The composition of the first aspect of the invention may comprise a skin lightener in addition to the compound of formula I and the agent. The additional skin lightener can include one or more of kojic acid, hydroquinone, arbutin or a plant extract containing arbutin such as Bearberry extract, glycyrrhizic acid or liquorice extracts containing it, protocatechuic acid and lactic acid.

In addition, the composition may contain other active ingredients with complementary activities to the compound of formula I and/or the other ingredients, such as antioxidants, anti-irritants, anti-microbials, anti-dandruff and anti-acne ingredients. The composition may also include actives responsible

for exfoliation (e.g. alpha- and beta-hydroxy acids), UV absorbtion, inhibition of collagenase/elastase (i.e. anti ageing) and stabilisation of labile antioxidants.

For the purposes of this invention, the agents can be replaced by a derivative of the agent which has comparible functional characteristics. Additionally, any isomer of each of the agents can be used in the composition. In particular, alpha-tocopherol can be replaced by any isomer of tocopherol, for example, alpha, beta, gamma or delta forms of D- or L-tocopherol, as discussed above.

Vinylguaiacol and/or ethylguaiacol may be provided in encapsulated form.

In a second aspect of the invention, there is provided the use of a compound of formula I and an agent in the manufacture of a composition for use as a lightener, wherein the agent decreases the cytotoxic effect of the compound of formula I on skin cells.

In accordance with the second aspect of the invention, the composition is provided as a hair or a skin lightener.

The preferred features of the first aspect of the invention, also apply to the second aspect.

The composition of the first or second aspects of the invention, is preferably provided as a formulation which is applied externally to the skin surface or to the hair. The composition can be in the form of a cream or lotion (including oil in water and water in oil creams and lotions), spray, powder, gel, shampoo, conditioner, mousse, serum, oil, stick or patch. The formulation can be provided specifically as a skin lightening product, or the skin lightening preparation can be added to a formulation for a different use (i.e. sunscreen lotion, day cream, moisturiser, perfume, body spray, bath or shower product).

The composition of the first or second aspects of the invention can be provided as a cosmetic product, a pharmaceutical product or a personal care product. For the purposes of this invention "cosmetic products" are products intended for increasing the appeal, visually and olfactively, of the human body. Likewise "personal care products" are products intended for cleaning, smoothing or otherwise improving the health and well-being of the outside of the human body. These definitions of cosmetic and personal care products at least partially overlap since many products provide functions in both categories. Examples of such products are: perfumes and like products known as "eau de toilette" and "eau de parfum", hand and body lotions, skin tonics, shaving products, bath and shower products, deodorant and antiperspirant products, hair care products such as shampoos and hair conditioners, mouth and dental care products. Such products are well known in the art. Thus, examples of skin care products are described in "Harry's Cosmeticology", R. G. Harry, 6th edition, Leonard Hill Books (1973), Chapters 5-13, 18 and 35; examples of deodorants and antiperspirants are described in C. Fox, cosmetics and Toiletries **100** (Dec. 1985), pp 27-41; examples of hair care products are described "Harry's Cosmeticololgy", vide supra, chapters 25-27; examples of dental care products are described in M. Pader, Oral Hygiene: Products and Practice, Marcel Dekker, New York (1988). Cosmetic and personal care products are usually perfumed, on the one hand to give pleasant odour to the products themselves and on the other hand to have the body parts to which they are applied emit a pleasant odour after their use.

The third aspect of the invention provides the use of an agent to decrease the cytotoxicity of the compound of formula I on skin cells. As previously discussed, the agent can decrease the cytotoxicity of the compound on skin cells either by increasing the dose of the compound required to only produce 50% cytotoxicity of skin cells and/or by increasing the efficacy and therefore

decreasing the dose of the compound required to produce 80% inhibition of melanogenesis.

All preferred features of the first and second aspects of the invention also apply to the third aspect.

A fourth aspect of the invention relates to a method for lightening the colour of skin or hair. This method comprises applying to skin or hair a composition comprising a compound of formula I and an agent which decreases the cytotoxic effect of the compound on skin cells. The method may be cosmetic or pharmaceutical.

The composition can be applied to the skin or hair daily, every other day, biweekly or weekly. The composition can be applied to the skin or hair as a lightening preparation or can be incorporated into a product with another use such as a moisturiser, sun screen, body spray, perfume, bath or shower product, shampoo or conditioner.

The compound of formula I and the agent can supplied as a composition with both components premixed. Alternatively, the components can be supplied separately and mixed prior to application. The components may also be supplied separately and applied sequentially.

All preferred aspects of the first, second third and fourth aspects of the invention apply to the fifth aspect.

The compounds of formula I can be provided from a number of sources. Preferably, the compounds are provided in the form of an extract derived from a plant material which has been subsequently treated with a micro-organism.

Vinylguaiacol and/or ethylguaiacol may be prepared by a bioprocess which comprises treating a substrate with one or more microorganisms selected from *Rhodotorula*, *Saccharomyces* (eg, *S.cerevisiae*), *Paecilomyces*, *Candida* and *Paenibacillus*; wherein the substrate is selected from ferulic acid or caffeic acid.

An advantageous feature of such bioprocesses is that they are natural, that is, they involve biological, especially enzymatic processes. Vinylguaiacol and ethylguaiacol are readily biodegradable because they occur in nature.

Skilled persons will appreciate that the bioprocesses are not limited to the specific examples, but include micro-organisms and/or enzymic and/or cell free extracts and/or engineered cells or enzymes therefrom which exhibit a suitable enzymic activity.

The micro-organism or enzyme or cell-free extract derived therefrom may produce the desired product efficiently and in high yields. This may be quantified in terms of: the rate of production of the product ($gl^{-1}day^{-1}$); the concentration of the product that accumulates (gl^{-1}); the yield of the product obtained from the substrate (g of product per g of substrate or % M yield); and the absence of side products which is reflected in the purity of the isolated product (% purity).

The strains may exhibit tolerance to high concentrations of both the substrate and the product, for example at least $1gl^{-1}$, preferably in the range of 1 to $40gl^{-1}$, more preferably in the range of 5 to $40gl^{-1}$. The strains may also exhibit high metabolic selectivity for the production of the required products, for example the products may be produced in at least 75% reaction molar yield and at least 50% recovered molar yield, and they have the ability to produce the products as non-growing cells so that, for example, expensive nutrients do not have to be supplied and expensive sterile fermentation equipment does not have to be used.

In particular, the criteria for establishing suitability of the micro-organism or enzyme or cell-free extract for use in the methods of the invention are as follows:

The micro-organism or enzyme or cell-free extract derived therefrom may produce at least 1g of the desired product per litre of reaction fluid and/or at least 50% molar yield of the desired product from the substrate (eg ferulic acid or caffeic acid) at a concentration of $>0.5gl^{-1}$. The desired product may have a purity of at least 90% as determined by positive characterisation of the product by *ab initio* analytical methods such as NMR.

The micro-organism or enzyme or cell-free extract may be capable of being used repeatedly, in two phase reaction systems, as immobilised cells, as disrupted cells, and is capable of reacting with impure substrates, especially plant extracts, if required.

Preferably, the bioprocess includes a biphasic reaction mixture. More preferably, the biphasic reaction mixture includes an aqueous phase, such as water, and a water immiscible (eg, organic liquid) phase such as vegetable oil, for example miglyol. The water immiscible phase acts as a product 'sink' in which the desired product formed from the substrate accumulates. This prevents accumulation of the product in the aqueous phase to levels which may inhibit or terminate the enzymatic reaction. This results in increased product yields compared to when the bioprocess is performed using a monophasic reaction mixture.

The product should be produced over a reasonably short period of time eg 1 to 3 days or less. Preferably, the test microorganism is isolated using the soil isolation protocol described hereinafter.

Preferably, one or more of the components of the composition are provided in the form of an extract from a plant material. Suitable extracts from plant materials include, for example, a maize extract containing vinylguaiacol (eg, in an amount of greater than 50%). In the compositions of the invention, vinylguaiacol and/or ethylguaiacol may be in encapsulated form.

Preferred embodiments of the invention will now be described with reference to the following illustrative examples.

Bioprocess for making compounds of formula I

In the following examples, analysis of vinylguaiacol and ethylguaiacol was carried out using high performance liquid chromatography (hplc) using the following conditions:

Column	-	Spherisorb C-18
Mobile phase	-	60:40 deionised water: MeCN; 1% acetic acid
Flow rate	-	2 mlmin $^{-1}$
Detection	-	Ultraviolet at 290 nm.

In the following examples, where organisms are grown in culture broth, the growth medium can contain specified amounts of either, or both, a vitamin supplement and a trace elements supplement. These were prepared as follows.

Vitamin supplement: biotin (2 mgl^{-1}), folic acid (2 mgl^{-1}), pyridoxine (10 mgl^{-1}), riboflavin (5 mgl^{-1}), thiamine (5 mgl^{-1}), nicotinic acid (5 mgl^{-1}), pantothenic acid (5 mgl^{-1}), vitamin B12 (0.1 mgl^{-1}), 4-aminobenzoic acid (5 mgl^{-1}), and thioacetic acid (5 mgl^{-1}).

Trace elements supplement: concentrated hydrochloric acid (51.3 ml^{-1}), MgO (10.75 gl^{-1}), $CaCO_3$ (2.0 gl^{-1}), $FeSO_4.7H_2O$ (4.5 gl^{-1}), $ZnSO_4.7H_2O$ (1.44 gl^{-1}), $MnSO_4.4H_2O$ (1.12 gl^{-1}), $CuSO_4.5H_2O$ (0.25 gl^{-1}), $CoSO_4.7H_2O$ (0.28 gl^{-1}), and H_3BO_3 (0.06 gl^{-1}).

Commercial supplies of *Sacharomyces cerevisiae* from Tesco plc, Sainsburys plc or Hovis yeast were used in Examples 2 and 8.

All other organisms were isolated using the soil isolation protocol described, unless indicated otherwise.

Patent Example	Identification of microorganism	Organism characteristics	Where isolated
1	*Rhodotorula glutinis* IMI 379894	Yeast with orange, mucoid colonies. Colony form: circular, entire margin convex elevation.	From air onto a yeast malt agar plate.
3	See example 1	See example 1	
4	See example 1	See example 1	
6	*Candida versitalis* NCYC 1433		
8	*Candida versitalis* NCYC 1433		
10, 11	*Paenibacillus polymyxa* IMI 382464	Gram positive, group 2 bacillus with oval, centrally positioned endospore and a thick, ridged coat	Bird nesting site at Dorking, UK
Abbreviations IMI - International Mycological Institute, Egham, Surrey, UK NCYC - National collection of Yeast Cultures, Norwich, UK			

Soil Isolation Protocol

To 2ml deionised water was added approximately 100 mg soil The resulting suspension was mixed thoroughly (vortex mixer) allowed to stand at room temperature (22° C for 1 hour followed by further mixing to distribute suspended

material. The macroscopic solids were allowed to settle for approximately 10 minutes and the supernatant (100µl) was applied to a suitable medium (see below) in a 90mm petri dish using a spread plate technique. Plates were incubated at 28°C until colony development was observed.

For the isolation of fungi, soil supernatants were spread plated onto a yeast malt medium comprising: 4g glucose, 4g yeast extracts, 10g malt extract per litre deionised water.

For the isolation of bacteria, soil supernatants were spread plated onto nutrient agar (Oxoid, Unipath Limited, UK)

Example 1: Preparation of Vinylguaiacol

A strain of *Rhodotorula glutinis* (IMI 379894) was cultured at 30°C by shaking at 200 rpm on a yeast malt medium containing (per litre of deionised water): glucose 4g; yeast extract 4g and malt extract 10g. After 40 hours incubation, ferulic acid was added to a final concentration of $2gl^{-1}$. The incubation was continued for a further 21 hours during which time the progress of the reaction was monitored by h.p.l.c. analysis using the conditions described above.

After 21 hours incubation the reaction had progressed to a molar conversion of 97.4%. The molar conversion after 3 hours was 61%.

Example 2: Preparation of Vinylguaiacol

Saccharomyces cerevisiae, "Bakers yeast", (2g, purchased from J. Sainsburys plc under the trade mark Sainsburys Easy Blend) was activated by suspending dry yeast powder in deionised water (20 ml) for 30 minutes at 37°C. A medium (1 l) containing (per litre deionised water): glucose 4g; yeast extract 4g and malt extract 10g was inoculated (5%) with the activated yeast suspension and incubated at 30°C with shaking at 200rpm for 96 hours. After 96 hours

incubation, the cells were harvested by centrifugation (15 minutes at 4000rpm), resuspended in 50ml of 0.9% (w/v) NaCl, and then disrupted by passage once through a cell disrupter (operating pressure 30,000psi). To 50ml of the resultant disrupted cell suspension was added ferulic acid at an initial concentration of $10gl^{-1}$. Also added at the same time was 50ml of Miglyol to form an upper organic layer to the biphasic biotransformation mixture. The progress of the reaction was monitored by analysis as described above. After incubation at 30°C for 64 hours the reaction had progressed to a 92% conversion to vinylguaiacol.

Example 3: Preparation of Vinylguaiacol from Maize Fibre

Ferulic acid was released from maize fibre as follows. A 10g portion of maize fibre was shaken (200 rpm) at 30°C, overnight, in a conical flask with 100 ml of 1M sodium hydroxide solution. The resulting solution was acidified to pH 5.5 prior to the addition of 45 ml of a culture of *Rhodotorula glutinis* (IMI 379894) which had been grown on yeast malt medium in a 250 ml shake flask and incubated with shaking (200 rpm) at 30°C for 40 hours. At this time a concentration of 0.495 gl^{-1} ferulic acid was detected. The resulting suspension was itself incubated at 30°C with shaking (200 rpm) and the vinylguaiacol concentration monitored by hplc. After 10 minutes a 7.9% conversion of ferulic acid to vinylguaiacol was observed; after 1 hour there was a 29% conversion; after 20 hours a 93% conversion. The reaction mixture was extracted twice with 50ml of n-hexane and the combined extracts dried and evaporated to yield 48mg of an oil comprising 84% vinylguaiacol.

Example 4: Preparation of Vinylguaiacol from Maize Fibre

Ferulic acid was released from maize fibre as follows. A 50g portion of maize fibre was shaken (200 rpm) at 30°C, for 15 hours, in a conical flask with 500 ml of 1M sodium hydroxide solution. The resulting solution containing 940 mg

ferulic acid was neutralised by the addition of concentrated hydrochloric acid. This was added to 1 litre of a culture of *Rhodotorula glutinis* (IMI 379894) which had been grown on yeast malt medium in a 5 litre shake flask and incubated with shaking (200 rpm) at 30°C for 24 hours. The mixture was adjusted to pH 5.5 and 1 litre of n-hexane was added. The resulting two-phase system was mixed gently (80 rpm) at 30°C. After 24 hours the two liquid phases were separated and the aqueous re-extracted with 500 ml of n-hexane. The combined organic solvent phases, which contained 540 mg vinylguaiacol (75% yield) were dried and evaporated to yield an oil (740 mg) which was 65% vinylguaiacol by assay. This represents a 66% recovery of vinylguaiacol from ferulic acid.

Example 5: Production of Ethylguaiacol from ferulic acid using C. versitalis

Candida versitalis (NCYC 1433) was grown from a plate culture inoculum for 6 days in yeast malt medium containing 10g/L malt extract, 4g/L yeast extract, 4g/L glucose, and 2% sodium chloride dissolved in deionised water and autoclaved at 120°C. The 50ml culture was incubated at 30°C and 200 rpm in a 250ml conical flask.

After 6 days this culture was used to provide a 10% inoculum for a 150ml second culture of the yeast malt medium occupying 50% v/v of the flask. This was incubated at 21-22°C for 24 hrs while agitating at 150 rpm. Then ferulic acid was added to a concentration of 2g/L, together with 100 ml of Miglyol, which alternatively could be added after 50 hrs when the concentration of ethylguaiacol in the aqueous phase had reached 0.25-0.3g/L. (Miglyol is added because the strain appears to be intolerant of the ethylguaiacol product, with the maximum concentration of ethylguaiacol accumulated (in a monophasic reaction) in the absence of Miglyol as product sink being 0.5 g/L).

Ethylguaiacol formation was monitored by hplc using as solvent 60:40 water:acetonitrile plus, 1% acetic acid, at a flow rate of 2ml/min and monitoring at 290 nm. Ethylguaiacol was formed in a good yield from ferulic acid, with vinylguaiacol being detected as the intermediate. After 184 hrs incubation, the concentration of ethylguaiacol in the Miglyol was 3.64g/L, which represents 92 to 94% of the theoretical maximum yield. The ethylguaiacol could be easily recovered from the Miglyol as a pure chemical by solvent extraction into hexane and then rotary evaporation to dryness.

Example 6: Reduction of vinylguaiacol to ethylguaiacol

Vinylguaiacol was produced from ferulic acid by microbial bioconversion as described in Example 1 or Example 2.

Vinylguaiacol (500 mg) and cobalt (II) sulfate heptahydrate (940 mg) were dissolved in ethanol (10 ml) under a helium atmosphere. Sodium borohydride (255 mg), dissolved in ethanol (5 ml), was added slowly with stirring in an ice bath. The dark solution was then permitted to stir at room temperature. After 36 hours an hplc trace showed that only about 4% of the starting material remained. A peak with the same retention time as ethylguaiacol (6.0 mins, 60:40 H_2O: MeCN + 1% AcOH) was present. The ethanolic solution was poured into 2M HCl (20ml) and extracted twice with diethyl ether. The organic layer was dried over sodium sulfate. Ethanol (100ml) was added to the diethyl ether and the latter was removed *in vacuo* to leave an ethanolic solution. Ethylguaiacol was recovered in a yield of 72% from the starting ferulic acid, and with no residual vinylguaiacol remaining in this recovered ethylguaiacol product.

Example 7: Production of vinylguaiacol in the presence of miglyol

Candida versitalis (Zyl 866; NCYC 1433) was grown (from a 10% inoculum) in 25ml of sterile yeast malt medium containing 2% w/v sodium chloride. After 24 hours ferulic acid was added to a final concentration of 4 g/l and 25 ml of

miglyol was also added as a supernatant. The flask was shaken at 250 rpm at 21°C and the solutions assayed by HPLC.

Over 134 hours the amount of vinylguaiacol increased in the miglyol phase of the reaction with relatively little vinylguaiacol being present in the aqueous phase of the bioconversion. There was also evidence of ethylguaiacol being present in the miglyol phase of the reaction. By comparison of peak areas on HPLC it was seen that use of 8 g/l ferulic acid in the reaction led to similar amounts of vinylguaiacol being produced whereas 2 g/l or 1 g/l ferulic acid in the reaction gave lower quantities of vinylguaiacol.

Table 1: Preparation of compounds of the invention from ferulic acid and selected microorganisms.

Product	Reaction molar yield % (g substrate supplied l^{-1})	Product g l^{-1} culture	Recovered molar yield (%)	Productivity of biocatalyst (g product g cells^{-1} dry wt.)	Approx. yield from 500l fermenter (kg)	Zyl No.
Ferulic acid to vinylguaiacol	94 (8.5)	6.1	87	0.95	2.4	702
Ferulic acid To Ethylguaiacol	92-94	3.64	n.d.	n.d.	0.73	NCYC 1433

Example 8: Preparation of vinylguaiacol from ferulic acid using **Paenibacillus polymyxa**

Paenibacillus polymyxa (Zyl 277; IMI CC Deposit No 382464) cells were grown at 30°C, shaking at 200 rpm for 27 hours on a medium comprising per litre deionised water: $(NH_4)_2SO_4$, 5g; K_2HPO_4, 2g; NaCl, 0.2g; glucose, 10g; malt extract, 3g; yeast extract, 3g; $MgSO_4$, 0.22g; $CaCl_2$, 0.015g; ferulic acid, 0.5g. The cells were harvested by centrifugation (4,000 x g 15 m') washed with 0.9% (w/v) saline solution followed by resuspension in 0.9%(w/v) saline solution as a

20-fold concentration. An aliquot of concentrated cells (5ml) was added to a solution of sodium alginate (15ml 3.5% w/v) and mixed thoroughly, prior to addition dropwise from a 3ml plastic pipette into 1 litre of 0.2M $CaCl_2$ solution. The beads formed by this procedure were stored at 4°C overnight in $CaCl_2$ solution to harden before washed in 2l of tap water.

The beads interspersed with an inert packing material were packed into a 100ml glass column. A solution of ferulic acid in tap water (500 ml, 6g/l) was pumped continuously through the column at a temperature of 24°C and the pH of this solution was maintained at pH 7.0. After 6 hours operation, the aqueous stream exiting the top of the column was continuously extracted into hexane (500 ml) to remove vinylguaiacol, prior to returning to the column.

Results

Vinylguaiacol concentrations (gl^{-1}) were:

Time (Hr)	Aqueous	Hexane	Total
1.75	0.074	-	0.074
3	0.116	-	0.116
6	0.175	-	0.175
23	0.101	0.8	0.901
48	0.144	1.332	1.476
122	0.152	2.09	2.24

Example 9: Production of Vinylguaiacol from Ferulic Acid by* Paenibacillus polymyxa *(ZYL277) in a Two Phase System

Paenibacillus polymyxa (Zyl 277; IMI CC Deposit No 382464) was grown in a bioreactor in a medium containing (g/l) $(NH_4)_2SO_4$, 5; K_2HPO_4, 2; NaCl, 0.2; yeast extract, 2; malt extract, 2; glucose, 10; ferulic acid, 0.5; 10ml/l of a

solution containing 0.1M $MgSO_4$/0.01M $CaCl_2$; at 30°C, pH 6.0, oxygen 70% on a stirrer cascade (100-500 rpm).

After 24h, 100 ml of culture was placed in a 250ml conical flask, stirred at 25°C with pH control at 7 using 2M NaOH or dilute phosphoric acid as required. 4 g/l ferulic acid (free acid) was added to the aqueous phase before it was overlaid with 100ml hexane. The hexane was added to partition vinylguaiacol from the aqueous phase where it may be toxic to the organism. Vinylguaiacol concentrations in both the aqueous and hexane phase were determined by HPLC. Further ferulic acid was added to the aqueous phase as the reaction proceeded. The hexane layer was removed periodically and replaced with 100ml of new hexane to prevent it becoming saturated with vinylguaiacol. Vinylguaiacol concentrations in both phases at the time of changing the hexane phase are shown below along with the cumulative total ferulic acid added to the aqueous phase (g/l).

Time (h)	Vinylguaiacol (g/l)			Total Ferulic Acid
	Aqueous	Hexane	Total	Added (g/l)
21	0.70	9.78	10.48	12
93	0.78	9.86	10.64	22
117	0.70	10.44	11.14	34
143	0.60	7.94	8.54	42
172	0.37	7.30	7.67	50
262	0.62	10.58	11.20	58
314	0.53	9.40	9.93	66

The seven collected hexane layers contained a total of 5.42g vinylguaiacol. Ferulic acid additions had been carried out to take account of increases in aqueous volume due to pH control. In total 7.16g ferulic acid had been added (equivalent to 66 g/l taking account of increasing aqueous volume). This equates to a molar yield for vinylguaiacol of 98%.

Example 10

Skin Lightener Assay Method:

Tyrosine: To 171.4µl of a solution of tyrosine (1.5mM) in phosphate buffer (100mM, pH6.4) was added of an inhibitor solution (100µg ml^{-1}) in phsophate buffer (100mM, pH6.4) in a quartz cuvette. The reaction mixture was made up to 980µl with phosphate buffer (100mM, pH6.4) and the reaction initiated by the addition of tyrosinase (Sigma, 220 µl, 1100 units/ml in phosphate buffer (100mM, pH6.4)), The reaction was monitored by the increase in absorbance at 470nm over 10 minutes.

Table 2

In vitro Skin Lightening Data

Tyrosinase Inhibitor	Degree of Inhibition IC_{50}* (µg/ml)
Vinylguaiacol	21.5
Ethylguaiacol	40
Hydroquinone +	2.27
Bearberry extract +	3.1
Bearberry extract +	4.4
Kojic acid +	6.5

*Concentration of inhibitor required reducing tyrosinase activity to 50%

+Standards

Melanogenesis inhibition (skin lightening) assays

These were conducted using cultured melanocyte cells exposed to dilutions of the best materials, and compared with kojic acid, a brown skin lightener. The degree of melanin production was inversely proportional to the skin lightener activity. Any cytotoxic effect of the test materials is reflected in a decrease in melanin formation.

Controls

Each 96 well assay plate included a solvent control and a blank control. Eight concentrations of kojic acid were used as a positive control.

Subculturing the cells into 96 well plates

When the cell cultures were 50 to 80% confluent, the growth medium was removed from the flasks and the cultures rinsed with 10 ml of HBSS. 2 ml of trypsin/EDTA solution was added and the flasks were incubated at 37°C +/- 1°C for 2 to 5 minutes. When the cells started to dislodge, the flask was rapped sharply against the palm of the hand and approximately 5 ml of assay medium added to neutralise the trypsin.

The concentration of cells was determined by counting an aliquot of the stock cell suspension in a haemocytometer. A seeding suspension of 5 X 10^4 cells/ml was prepared in assay medium. One hundred microlitres of the seeding cell suspension was added to the appropriate wells on each 96 well plate. One hundred microlitres of growth medium was added to the outer wells to maintain humidity. The cells were incubated at 37 +/- 1°C in a humidified atmosphere containing 5 +/- 1% CO_2 in air for 24 hours.

Skin lightening assay

Based on the information available for the test article, eight decreasing doses were selected and used in the assay. The highest dose of test article was a concentration of 5 mg/ml, based on its solubility in the solvent. The maximum solvent concentration (other than assay medium) was 1%.

The test article and positive control was tested by treating six wells per dilution of B16-F1 cells seeded approximately 24 hours earlier. Prior to treatment the medium was removed, and 200 µl of the pre-warmed (37 +/- 1°C) test article dilutions, positive control dilutions and solvent control, were added to the appropriate wells. All test article concentrations were dosed into a single outer

well (200 µl) of the corresponding plate to serve as a turbidity control. The remaining wells around the edge of the plate, designated as blanks, received 200 µl of assay medium prior to incubation. Following dosing, the plates were incubated at 37 +/- 1°C for 96 hours +/- 2 hours.

After exposure, 150 µl of each treatment solution (containing any secreted melanin) was removed and transferred to the corresponding wells of a new 96 well plate (melanin production plate). Turbidity controls were also transferred. The absorbance of the melanin present in the melanin production plates was measured at 405 nm (OD_{405}) with an Anthos 2010™ microplate reader.

The cells remaining in the assay plate were used to determine the cytotoxicity of the test article (cytotoxicity plate). The remaining media was removed and 100 µl of growth medium containing 25 µg/ml of neutral red was added to each well. The plates were returned to the incubator for 3 hours +/- 5 minutes, after which time the NR medium was decanted and the cells washed with 150 µl of PBS. The PBS was removed by gently tapping the plate. One hundred and fifty µl of NR desorb solution was added to each well. After a minimum of 20 minutes incubation at room temperature, the plates were agitated and the absorbance of the neutral red was measured at 540 nm (OD_{540}) with an Anthos 2010™ microplate reader.

Melanin Production

The mean of the six OD_{405} values for each concentration of test article and positive control was calculated. The turbidity control values were subtracted to obtain a correct OD_{405} value. The mean of the six OD_{405} values for the corresponding negative or solvent control were calculated by subtracting the blank control value. The corrected OD_{405} values of the test article or positive control was divided by the corrected OD_{405} value for the negative or solvent control to obtain a melanin production ratio.

Cytotoxicity

The mean of the six OD_{405} values for each concentration of test article and positive control was calculated. The blank control value was subtracted to obtain a corrected OD_{405} value. The mean of the six OD_{540} values for the corresponding negative or solvent control was calculated in the same way. The corrected OD_{540} values of the test article or positive control was divided by the corrected OD_{540} value for the negative or solvent control to obtain a cytotoxicity ratio.

Use of vinylguaiacol in combination with other agents

The efficacy and cytotoxicity of vinylguaiacol in combination with an agent (1:1 w/w) was investigated. The results are shown in Table 3.

Skin lightener	Dose for 80% inhibition of melanogenesis (mg/ml)	Dose for 50% cytotoxicity (mg/ml)	Difference between inhibition and cytotoxic doses (fold)	Improvement in cytotoxicity
Kojic (standard)	0.50	5.0	X 10	-
Vinylguaiacol	0.015	0.15	X 10	-
Vinylguaiacol + ferulic acid	0.015	0.5	X 30	X3
Vinylguaiacol + α-tocopherol	0.05	1.5-5.0	X 30 – 100	X3 – 10

Vinylguaiacol + ascorbate	0.05	0.5-1.5	X 10 - 30	X1 - 3

Results

Many common cosmetic ingredients showed no effect, acting solely as diluents of the vinylguaiacol skin lightener activity and not interfering with its action.

However, ferulic acid, α-tocopherol and ascorbic acid all had a positive effect on the cytotoxicity of the vinylguaiacol. This means that vinylguaiacol can be used at higher concentrations and therefore have a greater skin lightening effect without affecting cells or skin viability.

α-tocopherol and ascorbic acid have no skin lightening effects themselves and so act as diluents of the activity of the vinylguaiacol. Nevertheless despite the reduction in melanogenesis inhibition activity, the gap between the concentrations of vinylguaiacol that cause 80% inhibition of melanogenesis and 50% cytotoxicity is increased significantly.

Ferulic acid is itself a good inhibitor of melanogenesis and so a good improvement in the gap between the inhibition and cytotoxic effects is achieved without a reduction in the dose of active required to achieve 80% inhibition of melanogenesis.

Additional assays were carried out to investigate the efficacy and cytotoxicity of vinylguaiacol in combination with ferulic acid and alpha-tocopherol (as a 1:1:1 w/w mixture).

Sample	Concentration of Active (mg/ml)				
	1.5	0.5	0.15	0.05	0.015

Vinylguiaicol					
% inhibition of melanogenesis	(100)	(110)	100	85	63
% viable cells	1	4	101	111	95
Vinylguaiacol and Ferulic acid					
% inhibition of melanogenesis	(100)	(100)	100	92	78
% viable cells	0	13	121	103	95
Vinylguaiacol and α-tocopherol					
% inhibition of melanogenesis	(100)	97	78	60	32
% viable cells	2	120	119	98	80
Vinylguaiacol and ferulic acid and α-tocopherol					
% inhibition of melanogenesis	(100)	100	97	95	76
% viable cells	20	119	134	106	98

(Values in brackets indicate where all or part of the inhibition is due to cell death)

Thus, using the combination of vinylguaiacol, ferulic acid and α-tocopherol there is no significant reduction in the dose of vinylguaiacol required to achieve 80% inhibition of melanogenesis as compared to use of pure vinylguaiacol or to vinylguaiacol and ferulic acid. In addition, a significant improvement in

cytotoxicity is obtained such that the vinylguaiacol, ferulic acid and alpha-tocopherol combination exhibits 100% viable cells at concentrations at which vinylguaiacol alone causes almost complete cytotoxicity (e.g. 0.5mg/ml).

Further assays have indicated that mixed isomers of tocopherol i.e. mixtures of alpha, beta, gamma and/or delta forms of D- and/or L- tocopherol) also reduce the cytotoxicity of vinylguaiacol.

Experiments on vinylguaiacol in combination with ferulic acid and ascorbic acid also show reduced cytotoxicity of vinylguaiacol.

Compositions of the invention

For the purposes of this invention "cosmetic products" are products intended for increasing the appeal, visually and olfactively, of the human body. Likewise "personal care products" are products intended for cleaning, smoothing or otherwise improving the health and well-being of the outside of the human body. These definitions of cosmetic and personal care products at least partially overlap since many products provide functions in both categories. Examples of such products are: perfumes and like products known as "eau de toilette" and "eau de parfum", hand and body lotions, skin tonics, shaving products, bath and shower products, deodorant and antiperspirant products, hair care products such as shampoos and hair conditioners, mouth and dental care products. Such products are well known in the art. Thus, examples of skin care products are described in "Harry's Cosmeticology", R. G. Harry, 6th edition, Leonard Hill Books (1973), Chapters 5-13, 18 and 35; examples of deodorants and antiperspirants are described in C. Fox, cosmetics and Toiletries 100 (Dec. 1985), pp 27-41; examples of hair care products are described "Harry's Cosmeticololgy", vide supra, chapters 25-27; examples of dental care products are described in M. Pader, Oral Hygiene: Products and Practice, Marcel Dekker, New York (1988). Cosmetic and personal care products are usually perfumed, on the one hand to give pleasant odour to the products themselves and

on the other hand to have the body parts to which they are applied emit a pleasant odour after their use.

Moreover, the compounds of the invention may be used in food and beverage compositions.

Tables 4 to 6: Personal Care Products including the compounds of the invention

Table 4 : A photostable sunscreen lotion

Ingredient	% w/w
Glyceryl monomyristate	5.00
Cetyl alcohol	2.0
Butylmethoxy dibenzoylmethane	1.00
Isopropyl myristate	7.00
Oleoyl alcohol	3.00
Polysiloxane A	5.00
DEA cetylphosphate	3.00
Water, deionised	67.00
Propylene glycol	6.00
A composition of the invention	0.60
Fragrance	0.30

Table 5

All purpose dry skin cream

Ingredient	%w/w
Phase A (80°C)	
Glyceryl stearate SE	12.00

Stearyl stearate (Hetester 412)	4.00
Di-C 12-15 Alkyl fumarate (Marrix SF)	2.00
Ganex V220	2.00
Minno 21	6.00
Phase B (80°)	
Water, deionised	63.00
Glycerin	10.00
Phase C	
A composition of the invention	1.00

Add Phase A to Phase B. Mix at 80°C. Cool to 60°C and add Phase C. Cool to 50°C and package.

Table 6

Moisturiser with sun protection

Ingredient	%w/w
Phase A (45°C)	
Water deionised	63.40
Phase B (dry blend)	
Magnesium aluminium silicate (Veegum Reg.)	1.00
Xanthan Gum	0.50
Phase C (45°C and dissolve)	
Propylene glycol isoceteth-3 acetate (Hetester PHA)	10.00
Octyl methoxycinnamate	7.50
Octyldodecyl neopentanoate (Elefac 1-205)	5.00
Benzophenone-3	2.50
Minno 21	5.00

Octyl salicylate	5.00
Phase D	
A composition of the invention	0.10

Add phase B to phase A; disperse well and hold at 45°C. Then add phase C slowly. Hold at 45°C, mix well then add phase D. Cool and mix to 30°C and pack.

Example 9: Skin Lightener Cream Formulation

A formulation was produced containing a compound of the invention by formulating the following ingredients:

Water, Butylene glycol, Arbutin, PEG 8, Alcohol, Jojoba oil, Squalane, Dimethicone, Glycerin, Petrolatum, Pentaerythrityl tetraoctanoate, PEG-60 hydrogenated castor oil, Behenyl alcohol, Potassium carbomer, Butyl alcohol, Methylparaben, Octyl methoxycinnamate, Benzophenone-3, Compound of formula I, Xanthan gum, Tocopheryl acetate, Stearyl glycyrrhetinate, Panthenenyl ethyl ether, Ethylparaben, Cholesteryl hydroxystearate, Trisodium edta, Sodium hyaluronate, Sodium citrate, Sodium metabisulfite, Tocopherol, Iron oxides.

Example 10: Skin Whitener

Ingredients	% weight
Surfac GMS SE40	10.00
Mineral Oil	2.00
Waglinol 6014	2.00
Cetearyl Alcohol	2.00
DC 200/350	1.00

Nipasol M	0.10
Eusolex 6007	0.50
Water	To 100%
Propylene Glycol	5.00
Empicol LZV	0.60
Nervanaid BA2	0.10
Composition of formula I	2.00
Ascorbic Acid	0.10
Sodium Metabisulphite	0.15
Irgasan DP300	0.10
Fragrance	qs
Colour	qs

qs indicates that the components are added "to taste" i.e. to a preferred amount.

Micro-organism Deposits

Exemplary micro-organisms suitable for use in accordance with the present invention have been deposited for the purposes of patent procedures under the Budapest Treaty with the IMI Genetic Resource Reference Collection which is an International Depositary authority recognised under the Treaty. The address of the IMI Collection is CABI Bioscience UK Centre Egham, Genetic Resource Collection, Bakeham Lane, Egham, Surrey, England TW20 PTY telephone 01784 470111, fax 01491 829100, e-mail bioscience@cabi.org.

Micro-organisms	Strain Number	Date of acceptance for patent purposes	IMI CC Number
Paecilomyces variotti	ZYL 733	20 November 1998	379901
Rhodotorula glutinis	ZYL 702	20 November 1998	379894
Paenibacillus polymyxa	ZYL 277	24 January 2000	382464

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Email: bioscience@cabi.org ✦ Internet: http://www.cabi.org

Dear Sir

NOTIFICATION OF ACCEPTANCE OF A DEPOSIT
FOR THE PURPOSES OF PATENT PROCEDURE

Microorganism: *Paenibacillus polymyxa* IMI Number: 382464

Strain number: Zyl 277

The above designated microorganism, received on 24 January 2000 was accepted for deposit for patent purposes on 24 January 2000 in accordance with the terms and conditions set out in the Application form signed by you on 25 January 2000, a copy of which should have been retained by you.

Yours faithfully

PP. Director

International Mycological
Institute, Egham, UK.
Genetic Resources Collection
DATE

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Dear Sir

VIABILITY STATEMENT FOR THE PURPOSE OF PATENT PROCEDURE

Microorganism: *Paenibacillus polymyxa* IMI CC Number: 382464

Strain number: Zyl 277

The viability of the microorganism identified above was tested on 31 January 2000. On that date the said microorganism was viable.

Conditions under which the viability test has been performed (completed only if the test was negative).

Statement issued in accordance with microorganism deposits for the purposes of patent procedures under the Budapest Treaty, IMI Genetic Resource Reference Collection being an International Depositary authority recognized under the Treaty.

Yours faithfully

International Mycological
Genetic Resources Collection
DATE

Director

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Dear Sir

NOTIFICATION OF ACCEPTANCE OF A DEPOSIT FOR THE PURPOSES OF PATENT PROCEDURE

Microorganism: Unidentified IMI CC Number: 379897

Strain number: ZYL 768

The above designated microorganism, received on 20 November 1998 was accepted for deposit for patent purposes on 29 November 1998 in accordance with the terms and conditions set out in the Application form signed by you on 16 November 1998, a copy of which should have been retained by you.

Yours faithfully

Director

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Dear Sir

VIABILITY STATEMENT FOR THE PURPOSE OF PATENT PROCEDURE

Microorganism: Unidentified IMI CC Number:379897

Strain number: ZYL 768

The viability of the microorganism identified above was tested on 15 December 1998. On that date the said microorganism was viable.

Conditions under which the viability test has been performed (completed only if the test was negative).

Statement issued in accordance with microorganism deposits for the purposes of patent procedures under the Budapest Treaty, IMI Genetic Resource Reference Collection being an International Depositary authority recognized under the Treaty.

Yours faithfully

Director

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Dear Sir

NOTIFICATION OF ACCEPTANCE OF A DEPOSIT FOR THE PURPOSES OF PATENT PROCEDURE

Microorganism: *Paecilomyces variotii* IMI CC Number: 379901

Strain number: ZYL 733

The above designated microorganism, received on 20 November 1998 was accepted for deposit for patent purposes on 20 November 1998 in accordance with the terms and conditions set out in the Application form signed by you on 16 November 1998, a copy of which should have been retained by you.

Yours faithfully

Director

pp

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Dear Sir

VIABILITY STATEMENT FOR THE PURPOSE OF PATENT PROCEDURE

Microorganism: *Paecilomyces variotii* IMI CC Number: 379901

Strain number: ZYL 733

The viability of the microorganism identified above was tested on 25 January 1999. On that date the said microorganism was viable.

Conditions under which the viability test has been performed (completed only if the test was negative).

Statement issued in accordance with microorganism deposits for the purposes of patent procedures under the Budapest Treaty, IMI Genetic Resource Reference Collection being an International Depositary authority recognized under the Treaty.

Yours faithfully

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Dear Sir

NOTIFICATION OF ACCEPTANCE OF A DEPOSIT FOR THE PURPOSES OF PATENT PROCEDURE

Microorganism: *Rhodotorula glutinis* IMI CC Number: 379894

Strain number: ZYL 702

The above designated microorganism, received on 20 November 1998 was accepted for deposit for patent purposes on 20 November 1998 in accordance with the terms and conditions set out in the Application form signed by you on 16 November 1998, a copy of which should have been retained by you.

Yours faithfully

Director

pp

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Dear Sir

VIABILITY STATEMENT FOR THE PURPOSE OF PATENT PROCEDURE

Microorganism: *Rhodotorula glutinis* IMI CC Number: 379894

Strain number: ZYL 702

The viability of the microorganism identified above was tested on 22 December 1998. On that date the said microorganism was viable.

Conditions under which the viability test has been performed (completed only if the test was negative).

Statement issued in accordance with microorganism deposits for the purposes of patent procedures under the Budapest Treaty, IMI Genetic Resource Reference Collection being an International Depositary authority recognized under the Treaty.

Yours faithfully

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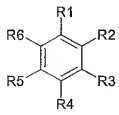
Director

CLAIMS

1. A composition comprising a compound of formula I



Formula I

and an agent wherein R^1 is $CH=CH_2$ or CH_2CH_3, R^3 is OMe, R^4 is OH and R^2, R^5 and R^6 are hydrogen, wherein the agent decreases the cytotoxic effect of a compound of formula I on skin cells.

2. A composition as claimed in claim 1 wherein the agent is an antioxidant.

3. A composition as claimed in claim 1 wherein the agent is an antioxidant and a skin lightener.

4. A composition as claimed in any one of claims 1 to 3 where the compound of formula I is vinylguauacol.

5. A composition as claimed in any one of claims 1 to 3 wherein the compound of formula I is ethylguaiacol.

6. A composition as claimed in any one of claims 1 to 5 comprising a further skin lightener

7. A composition as claimed in anyone of claims 1 to 6 wherein the agent is one or more of a tocopherol, ferulic acid or ascorbic acid.

8. A composition as claimed in any one of claims 1 to 7 comprising vinylguaiacol, α-tocopherol and ferulic acid.

9. A composition as claimed in anyone of claims 1 to 8 wherein the difference between inhibition of melanogenesis and cytotoxicity on skin cells of the composition is more than 10 fold.

10. A composition as claimed in anyone of claims 1 to 9 wherein the ratio of the compound of formula I to agent is about 100:1 to about 1:100.

11. A composition as claimed in claim 10 wherein the ratio of the compound of formula I to agent is from about 10:1 to about 1:10.

12. A composition as claimed in claim 11 wherein the ratio of the compound of formula I to agent is about 1:1.

13. A composition as claimed in anyone of claims 1 to 10 for use as a hair lightener.

14. A composition as claimed in claim 8 wherein the relative ratios of any two of the components vary from 10 to 1 fold.

15. A composition as claimed in anyone of claims 1 to 14 wherein the compound of formula I is provided in the form of an extract derived from a plant material which has been subsequently treated with a micro-organism.

16. The use of a compound of formula I and an agent in the manufacture of a composition for use as a skin lightener wherein the agent decreases the cytotoxic effect of the compound of formula I on skin cells.

17. The use as claimed in claim 16 wherein the agent is an antioxidant.

18. The use as claimed in claim 16 wherein the agent is an antioxidant and a skin lightener.

19. The use as claimed in anyone of claims 16 to 18 wherein the composition contains a further skin lightener.

20. The use as claimed in any one of claims 16 to 19 wherein the composition is a cosmetic composition.

21. The use as claimed in any one of claims 16 to 20 wherein the composition is a pharmaceutical composition.

22. The use as claimed in any one of claims 16 to 21 wherein the composition is applied topically to the skin.

23. The use of an agent to decrease the cytotoxicity of a compound of formula I on skin cells.

24. The use as claimed in claim 23 wherein the agent is an antioxidant.

25. The use as claimed in claim 23 wherein the agent is an antioxidant and a skin lightener.

26. The use of an agent as claimed in anyone of claims 23 to 25 in combination with a skin lightener.

27. The use of an agent as claimed in any one of claims 23 to 26 which is a cosmetic use.

28. A method for lightening the colour of skin comprising applying a composition as claimed in anyone of claims 1 to 16 to the skin.

29. A method as claimed in claim 28 which is cosmetic.

INTERNATIONAL SEARCH REPORT

In Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K7/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 00 47179 A (ZYLEPSIS LTD) 17 August 2000 (2000-08-17) claims 1-16; examples 13,14	1-29
P,X	WO 00 47045 A (ZYLEPSIS LTD) 17 August 2000 (2000-08-17) claims 1-11; example 19	1-29

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

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'O' document referring to an oral disclosure, use, exhibition or other means

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'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 January 2002	23/01/2002
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Willekens, G

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